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Involvement of multiple genetic loci in *Staphylococcus aureus* teicoplanin resistance

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Abstract

Teicoplanin resistance was transformed from a teicoplanin-resistant *Staphylococcus aureus* into the susceptible strain BB255 to give strain BB938. The cell wall composition, amidation of the iD-glutamate, and peptide crosslinking were identical in BB938 as in BB255 except for a 60% increased length of the glycan chain. Transductional crosses revealed that at least two distinct loci contributed in a cumulative fashion to teicoplanin resistance. One of these loci correlated with a mutation inactivating the anti-sigma factor RsbW. This mutation must have occurred during transformation and selection for teicoplanin resistance in BB938. Genetic manipulations involving the *sigB* operon showed that transcription factor SigB contributed to decreased teicoplanin susceptibility.

Keywords: Teicoplanin resistance; *sigB*; Pigment; Cell wall; *Staphylococcus aureus*

1. Introduction

Vancomycin and teicoplanin are the most commonly used drugs for the therapy of multiresistant, methicillin-resistant *Staphylococcus aureus*. They act by binding to the D-Ala–D-Ala termini of the nascent lipid II-linked murein precursor thereby inhibiting the polymerization of the glycan chains and the crosslinking of the peptide moiety of the peptidoglycan. Glycopeptide resistance in *S. aureus* can be produced through mutation and selection on exposure to glycopeptides. Teicoplanin resistance is more readily acquired than vancomycin resistance and emergence of teicoplanin resistance may be a prelude to emerging vancomycin resistance (reviewed in [1]). Among staphylococci, coagulase negative staphylococci became the first to have developed teicoplanin resistance. The first teicoplanin-resistant clinical *S. aureus* isolates emerging under teicoplanin therapy were described in 1990 [2]. Teicoplanin resistance was accompanied by an increase in the amount PBP2 and the production of a 35-kDa membrane protein [3]. A Tn*551* insertion in the *Sma*I-I fragment of the chromosome resulted in lowered PBP2 and 35-kDa membrane protein and increased sensitivity to teicoplanin. The 35-kDa protein was shown later not to be necessary for teicoplanin resistance [4], while PBP2 overproduction seemed to contribute to increased teicoplanin resistance in *S. aureus* [1]. Although increased vancomycin resistance results in increased teicoplanin resistance, the converse is not always true. In this study we have identified by genetic means one of the sites contributing to teicoplanin resistance.

2. Materials and methods

2.1. Bacterial strains and genetic manipulations

The strains used in this study are listed in Table 1. The strains were grown, where not mentioned otherwise, in LB medium (Difco, MI, USA) at 37°C. Transformation of BB255 was by the $CaCl_2$ method using early exponential phase cells resuspended in 0.1 M Tris–maleate buffer pH 7.5 containing 100 mM $CaCl_2$ as described by Novick [5]. Ten μg of chromosomal donor DNA in a volume of

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0.1 ml was added to 2 ml of competent cells. After 3 min in an ice water bath they were incubated for 3 min at 35°C, centrifuged and resuspended in growth medium for 1 h. The cells were harvested and resuspended into saline. Aliquots were plated with a soft agar overlay on LB plates containing 4 μg ml^{-1} of teicoplanin. The same numbers of cells treated with no DNA were used as controls. Transformants appearing after 2 days incubation were purified in presence of teicoplanin and analyzed by replica plating for their resistance profile. One colony with the highest teicoplanin minimal inhibitory concentration (MIC) of 16 mg ml^{-1}, strain BB938, was kept for further experiments. Transductions were done with phage 80α [6] grown on BB938. Transductants were selected with either 10 μg ml^{-1} of teicoplanin for first step, or 12 μg ml^{-1} for second step transductions, 20 μg ml^{-1} erythromycin, or 5 μg ml^{-1} tetracycline. Due to a strong inoculum effect on teicoplanin, the number of transformed or transduced cells plated on selective plates and the drug concentration had to be optimized. The large number of cells (10^9) plated on one single plate in transductional crosses required a selective concentration of 10 μg ml^{-1} of teicoplanin to prevent selection of spontaneous resistant mutants.

2.2. Resistance tests

Antibiotic MIC values were determined by the E-test method (AB Biodisk, Solna, Sweden) on BHI (Difco) agar according to the manufacturer's recommendation. Antibiotic susceptibility of different strains was compared on rectangular LB agar plates containing an antibiotic gradient. Population analysis profiles were made by plating 0.1-ml aliquots of different dilutions of overnight cultures grown in BHI on BHI agar containing increasing concentrations of teicoplanin. The colony forming units (cfu) were determined after 48 h incubation at 35°C. Methicillin was a gift from SmithKline Beecham Pharmaceuticals, and Hoechst Marion Roussel AG kindly provided teicoplanin.

2.3. DNA manipulations

Routine DNA manipulations were done according to protocols of Sambrook et al. and Maniatis et al. [7,8]. Separation of *Sma*I-digested chromosomal DNA by pulsed-field gel electrophoresis was performed as described by Wada [9]. PCR amplification of the *rsbU* gene to identify the 11-bp deletion in 8325 derivatives was done with the conditions described earlier [10]. Amplification of the *rsbW* gene region was done with the *rsbU*-specific forward primer 5′-CGTGGAAGAATTTAAGCAAC-3′ and the *rsbW*-specific reverse primer 5′-GCTGATTTCGACTCTTTCGC-3′ using the PWO polymerase (Roche Biochemicals, Rotkreuz, Switzerland). Sequencing with the BigDye (PE Biosystems) and ABI PRISM 310 Genetic Analyzers (PE Biosystems) was performed according to the manufacturer's recommendations.

2.4. Western blots

Western blot analysis was performed in accordance with Sambrook et al. [7], using antigen-purified polyclonal rabbit anti-RsbU and anti-RsbW antibodies (Giachino, P. and Bischoff, M., unpublished).

2.5. Peptidoglycan analysis

Extraction of cell walls, amino acid determination of cell wall material, and analysis of muramidase-digested peptidoglycan by reversed-phase high performance liquid chromatography (HPLC) (RP-HPLC) were done as described by Roos et al. [11]. Peptidoglycan crosslinking was calculated from the amounts of muropeptide monomer, dimer, trimer and oligomers in muramidase-digested cell walls, separated by RP-HPLC, as described by Dezelee et al. [12]. To determine the amount of free reducing termini of muramic acid and glucosamine, the peptidoglycan was reduced with sodium borohydride and hydrolyzed. The reduced and not reduced amino sugars were submitted to precolumn derivatization with the OPA/AcOH system (*ortho*-phthaldialdehyde/*N*-acetyl-L-cysteine). The resulting isoindol derivatives were separated by RP-HPLC and quantified by fluorescence detection [13]. The amount of reduced amino sugar divided by the total amount of amino sugars gave the percentage of free termini of the glycan chain [14].

Table 1
S. aureus strains

Strains	Relevant genotype or phenotype	Origin, remarks
14-4	resistance to methicillin, ampicillin, aminoglycosides, macrolides, chloramphenicol, tetracycline, and teicoplanin, pigmented colonies	teicoplanin-resistant subclone of the clinical MRSA strain MRGR3 (P. Vaudaux)
BB255	essentially the same as 8325, *pig*, *rsbU*, white colonies, transformable by the $CaCl_2$ method	[6]
BB938	BB255, *rsbU, rsbW*, resistant to teicoplanin, orange colonies	this study, by transformation of BB255 with strain 14-4 DNA and selection for teicoplanin resistance
PG223	BB938, Δ*(rsbUVW-sigB)::ermB*, teicoplanin-susceptible, white colonies	this study, by transduction of Δ*(rsbUVW-sigB)::ermB* from IK181 [10] into BB938
GP274	PG223, (*rsbU*$^+$*V*$^+$*W*$^+$-*sigB*$^+$)-*tet*, orange colonies, teicoplanin-resistant	this study, by transduction of (*rsbU*$^+$V$^+$W$^+$-*sigB*$^+$)-*tet* from GP267 into PG223 (Giachino, P. and Bischoff, M., unpublished)

3. Results

3.1. Strain construction

The teicoplanin-resistant strain 14-4 was derived from a multiresistant, initially teicoplanin-susceptible clinical isolate MRGR3 [15] which had been passaged in tissue cages implanted in Wistar rats [16]. Plating of MRGR3 ex vivo from untreated animals onto selective medium yielded a few colonies with increased teicoplanin resistance. Serial passage of bacteria from these colonies on teicoplanin yielded one mutant strain 14-4 that stably expressed teicoplanin resistance with an MIC of 24 μg ml^{-1}. Strain 14-4 is resistant to multiple antibiotics including methicillin, macrolides and aminoglycosides, and resistant to transducing phages. In order to study teicoplanin resistance in a defined genetic background, the teicoplanin resistance was transformed from strain 14-4 into the susceptible strain BB255, which is essentially the same as the genetically well characterized strain NCTC8325, which forms white colonies due to the *pig* mutation in the *Sma*I-I fragment [17]. Selection was for transformants able to grow in presence of teicoplanin. Mock transformations with no DNA gave no colonies on teicoplanin agar, but 10 colonies were obtained in the transformation. Most transformants acquired concomitantly with teicoplanin resistance an intense yellow–orange pigmentation. No other resistance was transferred. Their teicoplanin MICs ranged from 6 to 24 μg ml^{-1}. One yellow–orange transformant, BB938, with a teicoplanin MIC of 24 μg ml^{-1} was selected for further studies. Strain BB938 had the same teicoplanin resistance profile in a population analysis as that of the donor 14-4 (Fig. 1). *Sma*I restriction patterns of the BB255 and BB938 chromosomes were indistinguishable, but distinct from that of donor 14-4 showing that BB938 had retained essentially the BB255 background during transformation. Teicoplanin resistance of BB938 was increased but vancomycin resistance was very similar to BB255 (Fig. 2).

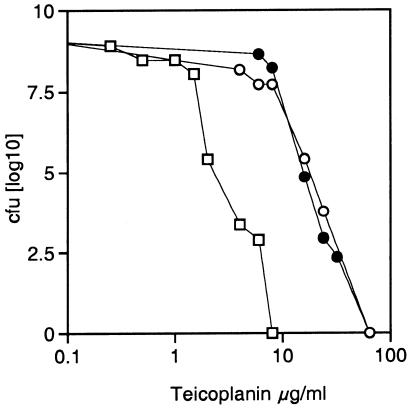

Fig. 1. Population analysis profile of recipient, donor, and transductant on teicoplanin. Squares, strain BB255; empty circles, strain 14-4; filled circles, strain BB938.

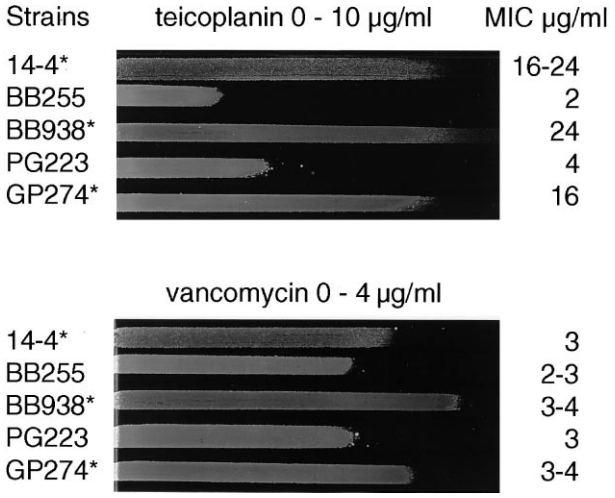

Fig. 2. Growth of strains on plates containing a gradient of teicoplanin or vancomycin. The corresponding MIC values of teicoplanin or vancomycin are indicated on the right side. An asterisk marks orange pigmented strains.

3.2. Peptidoglycan composition

Isolated cell walls of BB255 and BB938 were indistinguishable in amino acid content, sugar composition, and in the amidation of the iD-glutamate of the peptidoglycan stem peptide (data not shown). The peptidoglycan composition corresponded to that of normal *S. aureus* cell walls [11]. Similarly, the overall crosslinking of the peptide moiety, 74.4% in BB255, and 74.9% in BB938, showed no significant difference. The only difference was an increase in the length of the glycan chains in the teicoplanin-resistant strain. Strain BB255 showed an average of 34 monosaccharide units per glycan chain, while BB938 contained 52 monosaccharide units. Peptidoglycan synthesis results in glycan chains consisting of repeating units of the disaccharide (GlcNAc-(β-1,4)-MurNAc) with reducing termini in the muramic acid (MurNAc) residues. Reducing termini containing glucosamine (GlcNAc) are products of a glucosaminidase [18]. While in BB255 1.4% reducing termini ending with muramic acid, and 1.5% with glucosamine were found (total amount of 2.9% reduced termini), the corresponding values in BB938 were 1.6% reducing termini ending with muramic acid, and only 0.3% ending with glucosamine (total amount of 1.9% reduced termini). Reduced termini of glucosamine in *S. aureus* strains are usually in the range of 1.4–1.6%, with the exception of BB938 with only 0.3% reduced termini of glucosamine. This decrease in glucosamine containing endgroups in BB938 compared to BB255 may be the result of a lowered glucosaminidase activity. Moreover, analysis of the peaks of the RP-HPLC of muramidase-digested peptidoglycan showed peak areas for peptidoglycan constituents produced by the autolysins (endopeptidase, amidase and glucosaminidase) to be 30% lower in BB938 than in BB255.

3.3. Genetic sites involved in teicoplanin resistance

To identify the number of sites involved in teicoplanin resistance, transducing phage 80α was propagated on strain BB938 for back-transductions into BB255. We were unable to transfer the full teicoplanin resistance by a one-step transduction into BB255. The yellow–orange pigmentation was found to be 60% cotransducible with teicoplanin resistance, suggesting that teicoplanin resistance may be located close to the *pig* locus in strain BB255, since transducing phages are able to package approximately 42 kb of DNA [19]. None of transductants obtained was as resistant as BB938; all had lower teicoplanin MICs ranging between 4 and 8 μg ml^{-1}. Only a second transduction produced transductants with the original resistance of 24 μg ml^{-1} teicoplanin. The two transduction steps needed to transfer the original teicoplanin resistance suggested that at least two distinct loci were responsible for teicoplanin resistance in BB938.

3.4. Pigment production and teicoplanin resistance

It is known that pigment production is dependent on the activity of the transcription factor SigB encoded in the chromosomal fragment *Sma*I-I. Strain BB255 produces white colonies because of a deletion in *rsbU*, the first gene in the *sigB* operon *rsbUVW-sigB* [10]. The possibility that a mutation in the *sigB* operon was responsible for the intense pigment formation in BB938 was investigated as was the possible coupling to a gene conferring resistance. The entire *sigB* operon (Fig. 3C) was deleted in BB938 by transduction of Δ*(rsbUVW-sigB)::ermB* into BB938 with selection for erythromycin resistance. All transductants were white and had a lower MIC to teicoplanin, as shown by the representative transductant PG223 in Fig. 2. The resistance levels of PG223 were reduced to those of the first step BB255 transductants obtained earlier. In a further transduction, the *erm*-tagged deletion of PG223 was replaced by a wild-type *sigB* operon (*rsbU*$^+$*V*$^+$*W*$^+$-*sigB*$^+$) coupled to a *tet* resistance gene derived from the teicoplanin-susceptible strain GP267 (Giachino, P. and Bischoff, M., unpublished). In the tetracycline-resistant transductants obtained, pigment production was restored and the transductants were more resistant to teicoplanin (see GP274 – Fig. 2). This suggested a link between the *sigB* operon and teicoplanin resistance. Interestingly the teicoplanin MIC was slightly lower in GP274 (16 μg ml^{-1}) than for BB938 (16–24 μg ml^{-1}), suggesting that after the two transductions there were still some differences between GP274 and BB938.

The RsbU and RsbW gene products of the *sigB* operon in strains BB255, 14-4, and BB938 were analyzed by Western blots, with polyclonal anti-RsbU and polyclonal anti-RsbW antibodies. The unrelated strain Newman [20] was used as control, and the Western blot revealed RsbU and RsbW proteins which were also found in the teicoplanin-resistant strain 14-4. Strain BB255 produced no RsbU as expected from its *rsbU* deletion. BB938 was still devoid of RsbU (Fig. 3A) but had also lost RsbW (Fig. 3B). Loss of the anti-sigma factor RsbW would leave SigB uncomplexed and thus active, which could explain the synthesis of pigments in BB938.

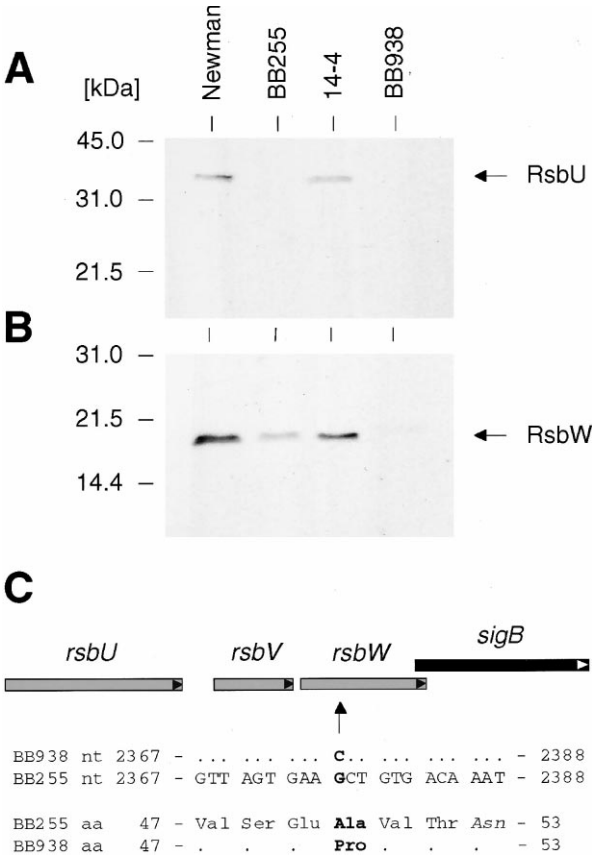

Fig. 3. Western blots of RsbU and RsbW and map of the *sigB* operon. (A and B) Ten μg of cytoplasmic proteins obtained from an overnight culture were subjected to Western blot analysis. Staining of the proteins with amido-black prior to antibody incubation confirmed equal loading of the lanes. Strain Newman was used as an unrelated control wild-type strain. Western blot, (A) with with polyclonal anti-RsbU antibodies; (B) with polyclonal anti-RsbW antibodies. (C) Map of the *sigB* operon [22] and nucleotide and amino acid changes due to the *rsbW* mutation in BB938.

Amplification of the DNA region across the 11-bp *rsbU* deletion confirmed that the 11 bp were deleted in both, BB255 and BB938 (data not shown). Sequencing of the chromosomal region of the *rsbW* gene of BB938 revealed a point mutation (G2367C, corresponding to accession number Y07645) within the translated region of *rsbW*, which would result in an amino acid substitution (A50P, corresponding to accession number CAA68931) in the anti-sigma factor RsbW (Fig. 3C). This mutation was localized to a region postulated to be essential for sigma factor interaction [21]. Since no RsbW was detectable in BB938 (Fig. 3B), although significant amounts of the *rsbV-rsbW-sigB* transcripts were seen in Northern blots (data not shown), an increased degradation of the mutated

RsbW was postulated. Instability of RsbW may either be due to misfolding, or to the inability to interact with SigB. Analogous observations have been made with the closely related anti-sigma factor SpoIIAB, that was found to be rapidly degraded when uncomplexed [21].

4. Discussion

Teicoplanin resistance was initially transformed from 14-4 into BB255 yielding a teicoplanin-resistant strain BB938. None of the other resistance determinants of 14-4 were cotransformed, presumably because they were located on plasmids or integrated in the *mec*-associated DNA in strain 14-4, while the genes affecting teicoplanin resistance were localized elsewhere on the chromosome. Full teicoplanin resistance could only be transduced in two steps, showing that at least two genetic loci were responsible for the teicoplanin resistance, of which one cotransduced with the *sigB* operon. Since the donor strain had a wild-type *sigB* operon, but the recipient BB255, as well as the resulting transformant BB938 had the 11-bp deletion in *rsbU*, the *rsbW* inactivation must have occurred during the selection of BB938 on teicoplanin, and was thus not acquired from 14-4 by transformation.

A correlation of the *sigB* operon with teicoplanin resistance became evident through the transduction of the intact *sigB* operon, from a susceptible donor GP267, resulting in an increase in resistance in PG223. Therefore SigB activity seems to play an important role in developing teicoplanin resistance. The slight differences in teicoplanin resistance levels between BB938 and GP274 could be explained by the fact that, in the former SigB had escaped anti-sigma factor RsbW interactions and thus was increased in activity, while in the latter SigB activity was controlled by RsbU, RsbV and RsbW. Alternatively a further gene contributing to teicoplanin resistance and mapping close to the *sigB* operon could not be ruled out. Such a gene may have been substituted by the wild-type allele in the transductions leading to PG223 and GP274. Moreover, the increased pigment content itself may confer some protective effect against teicoplanin through capturing of possible reactive agents. These hypotheses need to be investigated further. Interestingly, in a clinical teicoplanin intermediate-resistant isolate [3] a Tn*551* insertion in the *Sma*I-I fragment increased teicoplanin susceptibility and decreased PBP2 production, leading to the hypothesis that a regulatory DNA fragment was inactivated.

The increased glycan chain length in BB938 was postulated to be due to a decreased glucosaminidase activity in these strains. The impact of this observation in relation to teicoplanin resistance has to be investigated further. A glucosaminidase activity that modifies the *S. aureus* glycan structure in vivo has recently been demonstrated [18]. Teicoplanin resistance seems thus to be multifactorial, positively influenced by SigB activity, and additive.

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